Review Article

Contributions of DNA methylation aberrancies in shaping the cancer epigenome

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Abstract: Genetic alterations, including DNA sequence and copy number alterations, have been characterized in human cancers for over several decades. However, it is becoming increasingly evident that in addition to these events, epigenetic changes are widespread in virtually every cancer type. The scientific literature is rich with reports of changes in DNA methylation, chromatin modifications and nucleosome occupancy, all of which contribute to the aberrant gene expression profiles described in human cancers. Whilst genetic and epigenetic alterations have been historically characterized on a candidate gene and cancer-type basis, the unprecedented acceleration in microarray and next-generation sequencing technologies used to profile large collections of primary specimens has provided a panoramic, genome-wide view of cancer genomes and epigenomes. For the first time, the relationships between individual alterations can be understood, with the ultimate goal of improving cancer detection, monitoring, surveillance and most importantly, treatment. A central aspect of these involves characterizing DNA methylation alterations in human cancers. DNA methylation aberrancies are present in every tumor type, serve as stable biomarkers of disease, are associated with patient clinical features, and are reversible through the application of DNA methylation inhibitors. DNA methylation inhibitors are currently used in treating patients with myelodysplastic syndromes (MDSs) and acute myeloid leukemia (AML), and new inhibitors are currently in clinical trials for potential cancer treatment. This review highlights DNA methylation changes and their significance in human cancers from mechanistic, biomarker and treatment perspectives.

Keywords: DNA methylation; cancer; CpG island methylator phenotype (CIMP); chromatin; gene expression

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DNA methylation in mammalian tissues

DNA methylation is an important gene expression regulator, and serves as a guiding force for X chromosome inactivation, cellular differentiation and development, genomic imprinting and the suppression of repetitive elements. In mammalian cells, DNA methylation is mainly restricted to the C-5 position of cytosine in the 5'-CG-3', or CpG, sequence context, and are stable with successive rounds of cell division [reviewed in (1,2)]. DNA methylation in non-CpG regions, CpA, CpC or CpT (generically labeled as CpH), is present in embryonic stem (ES) cells (3). DNA methylation profiles are erased during in embryonic development, and then are re-established as cells develop towards the differentiated, somatic state [reviewed in (4)].

Establishing and maintaining CpG methylation patterns in somatic mammalian cells is accomplished by several DNA methyltransferases (DNMTs) and the co-factor *S*-adenosylmethionine. DNMT1 is historically considered as a maintenance methyltransferase, as it has an affinity for hemi-methylated DNA and is tightly coordinated to DNA replication machinery [reviewed in (1)]. DNMT3A and DNMT3B were identified as *de novo* methyltransferases, however, the coordinated efforts

of DNMT1, DNMT3A and DNM3B are thought to be required for establishing replicating existing DNA methylation patterns in cancer cells (1). DNMT3A and DNMT3B are believed to selectively anchor nucleosomes containing methylated CpG islands and repetitive elements, suggesting that DNA methylation patterns are influenced by DNA hemimethylation and chromatin cues (5,6). Finally, DNMT3L is only expressed during gametogenesis and embryonic development and serves as a scaffold protein in connecting DNMT3A to nucleosomes (7,8), while DNMT2 functions as a tRNA-methyltransferase (9,10).

Although CpG methylation is an essential regulatory element, it is inherently mutagenic, as 5-methylcytosine (5-mC) undergoes spontaneous deamination to thymine. The rate of 5-mC deamination is approximately an order of magnitude greater than the deamination of unmethylated cytosine to uracil (11-13). As a consequence, CpG content is 20% of what is expected. In addition, approximately 70% of CpG dinucleotides are generally methylated in normal somatic human tissues, representing 4-5% of all cytosines in the human genome, and are generally localized to repetitive elements and regions of low CpG density [reviewed in (12)]. Alternatively, there are regions of the genome, termed CpG islands, which contain their expected number of CpG nucleotides and G:C content. These typically are unmethylated in normal somatic tissues and are frequently located in gene promoter and 5' coding regions (12).

5-hydroxymethylcytosine and DNA demethylation

5-hydroxymethylation (5-hmC) was first described as a product of 5-mC oxidation by TET1 (ten-eleven translocase) [reviewed in (14,15)]. Two additional TET enzymes, TET2 and TET3, were subsequently identified, with each TET enzyme functioning as 2-oxoglutarate- and iron-dependent dioxygenases that are similar in function to several known histone lysine demethylases. TET enzymes can catalyze the conversion of 5-mC to not only 5-hmC, but also the subsequent conversion of 5-hmC to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). The latter (5-fC and 5-caC) are substrates for thymine DNA glycosylase-mediated base excision repair that results with replacement of the 5-fC and 5-caC base by an unmethylated cytosine. 5-hmC is present at lower levels (<1%) than 5-mC (4-5%), but 5-hmC marks are found at gene promoters, gene bodies and enhancers across tissue types. Specifically, the highest 5-hmC levels are found in brain, colorectal, kidney and liver tissues, whereas 5-hmC levels are substantially lower in heart and breast tissues (16). 5-hmC profiles are also altered in several human cancers [reviewed in (17)], and represent an important step in enzyme-catalyzed DNA demethylation, as well as potential cancer-specific biomarkers (18-22).

Interplay of DNA methylation and chromatin modifications in regulating gene expression

DNA methylation is closely associated with chromatin structure and nucleosome accessibility in regulating gene expression, and as a result, chromatin modifications are also altered in human cancers. Chromatin structure is predominantly described by post-translational modifications of specific amino acids on histone N-terminal tails, in which histone methyltransferases (HMTs), histone acetyltransferases (HATs), histone phosphorylases, and other enzymes catalyze the recognition (readers), addition (writers), removal (erasers) of these functional groups, thereby influencing chromatin structure, and ultimately, gene activity potential [reviewed in (23)]. Chromatin modifications also delineate between genes that display inducible or tissue-specific expression profiles and genes that display constitutive expression and unexpressed genes. Histone lysine monomethylation (H3K4, H3K9, H3K27, H3K79, H4K20) and acetylation (H3K9Ac, H3K14Ac and H3K27Ac) marks correlate with unmethylated DNA and active gene expression. Histone marks associated with gene activation include histone H3 lysine 4 tri-methylation (H3K4me3), H3K36me3 and H3K79me2, while repressed regions of the genome are enriched for H3K9me2, H3K9me3, H3K27me2 and H3K27me3 marks (23-25), which positively correlate with DNA methylation in gene promoters and repetitive elements. Ultimately, the key roles of DNA methylation in regulating chromatin structure are in stabilizing nucleosome position and acting as a repressive mark (1).

DNA methylation alterations in human cancers

DNA methylation alterations are widespread and present in every human cancer type. Specifically, human cancer methylomes generally display global DNA hypomethylation, especially with respect to repetitive elements, low-density CpG regions and lamin-attachment domains (26-34). These DNA hypomethylation events are concomitant with DNA hypermethylation at CpG islands and CpG island shores, which are defined as

the regions flanking CpG islands. CpG island shore methylation is also involved in both tissue-specific and cancer-specific differentially methylated regions (35). DNA hypermethylation of promoter/5' CpG islands can correlate with reduction in gene expression, mainly via recruitment of methylated DNA binding proteins (MBDs), together with specific chromatin modifications that result in closed, inactive chromatin marked by H3K27me3 [reviewed in (1,23,36,37)]. Alternatively, gene body DNA methylation correlates with increased gene expression and the presence of H3K36me3 across the gene body region (38-40).

Interestingly, cancer-associated DNA methylation is enriched at genes highlighted for transcriptional repression in embryonic stem (ES) cells via occupancy of the polycomb repressive complex 2 (PRC2), which consists of suppressor of zeste 12 (SUZ12), embryonic ectoderm development (EED) and H3K27me3 marks as a result of enhancer of zeste homolog 2 (EZH2) activity (41,42). These findings suggest a potential stem cell origin of cancer, in which the reversible repression of gene expression in ES cells is ultimately converted into an irreversibly silenced state by DNA hypermethylation and repressive chromatin marks (42). Indeed, tumors are thought to arise from early progenitor, stem-cell like or undifferentiated somatic cells, with activation of pathways related to progenitor/precursor cell growth (43), suggesting that tumors may acquire changes consistent with embryonic cells.

Epigenetic silencing is one mechanism by which genes encoding for tumor suppressors, DNA repair enzymes, and proteins involved in other cellular/regulatory pathways, are inactivated in human cancers. DNA methylation is included with somatic mutations and copy number alterations as means by which individual gene alleles can be inactivated in human cancers. The genome-wide scope of DNA methylation perturbations indicates that these are early events in human carcinogenesis. However, it should be noted that DNA methylation-mediated gene silencing occurs in a small proportion of genes in the cancer methylome. Indeed, less than 10% of hypermethylated genes displayed epigenetic silencing in several reports (44,45). However, this may also be a consequence of tumor cell heterogeneity. Epigenetic silencing may occur in a higher percentage of tumor cells, but may be masked due to both high tumor cell heterogeneity and technological limitations of characterizing single-cell epigenetic silencing events in primary tumors. The advancement of technologies to identify epigenetic silencing events in individual tumor cells will be critical for a complete understanding of the scope of epigenetic silencing as a driver event in human cancers.

In contrast to somatic mutations, DNA methylation alterations are substantially more abundant in human cancers, with approximately 400 hypermethylated genes per cancer genome (46), and therefore represent an abundance of biomarkers for cancer diagnosis, response to treatment and disease monitoring, as well as targets for epigenetic therapies. The latter is an important point, as DNA methylation is reversible through the use of DNA methylation inhibitors. While DNA methylation patterns are heterogeneous within individual loci as well as between individual cells, several gene regions display high frequencies of cancer-associated DNA hypermethylation, therefore allowing for high cancer detection sensitivity and specificity. In addition, while most studies in the field have focused on epigenetic silencing during tumorigenesis, gene body DNA hypermethylation may also be involved in up-regulation of genes in the MYC and metabolic pathways (40).

Candidate epigenetic driver genes and affected signaling pathways

An important aspect of cancer genomics is characterizing specific alterations that drive tumor formation, maintenance and progression. This is especially true with respect to cancer epigenetics, in which DNA methylation aberrancies are widespread in every cancer type. One approach of characterizing potential epigenetic driver genes is to identify genes that are epigenetically silenced in tumor types and subtypes. Epigenetic silencing of key tumor suppressor, regulatory and repair genes has been demonstrated in several cancer types, and as a result, several important cellular signaling pathways are also disrupted. These include DNA repair, RB1/CDK4 cell cycle regulation, WNT/β-catenin, TGF-β, cellular differentiation pathways, as well as others.

CDKN2A ($p16^{INK4A}$) is frequently silenced via promoter DNA hypermethylation across several human cancers (47,48). The p16 protein binds to cyclin-dependent kinases CDK4 and CDK6, which in turn blocks phosphorylation of retinoblastoma 1 (RB1), thereby allowing the cell to pass through the G1/S cell cycle checkpoint. In this manner, p16 also blocks improper cellular division as a result of DNA damage or oncogenic signaling, however, improper p16 function, whether the result of mutation, deletion or epigenetic silencing, allows the cell to bypass this checkpoint [reviewed in (49)]. For this reason, p16

is considered a tumor suppressor and p16 alterations are thought to be early events in tumorigenesis.

The evolutionarily conserved WNT/β-catenin signaling pathway, commonly referred to as the canonical WNT pathway, is frequently dysregulated in several forms of human cancer. WNT pathway alterations were identified in over 90% of colorectal tumors (50). WNT signaling is predicated upon a multi-protein complex comprised of APC, GSK-3β, axin and β-catenin. In the absence of WNT ligand, binding of WNT ligands to targeted Frizzled receptors leads to activation of the Disheveled protein and GSK-3β inhibition, which stabilizes β-catenin levels [reviewed in (51)]. As a result, β-catenin is localized to the nucleus and consequently binds to transcription factors that in turn induce expression of *MYC* and *CCND1*. WNT signaling is inhibited by the absence of ligand, or by the direct binding of WNT antagonists, including WNT inhibitory factors (WIFs) and secreted frizzled-related proteins (SFRPs) to WNT ligands of Frizzled receptors. SFRPs also inhibit cell cycle progression and cellular proliferation. Interestingly, several members of the *SFRP* gene family, namely *SFRP1*, *SFRP2*, *SFRP4* and *SFRP5*, are epigenetically silenced via promoter DNA hypermethylation in several cancer types, most notably in colorectal cancer (52,53).

Mutations in the *MSH* family of DNA mismatch repair genes are frequent events in human hereditary colorectal cancers (Lynch syndrome), thereby rendering the cancer genome susceptible to mutational burden and microsatellite instability (54). In addition, the mismatch repair gene *MLH1* is silenced by promoter DNA hypermethylation in a subset of sporadic colorectal cancers, resulting in the similar impairment of DNA mismatch repair and subsequent expansion of microsatellite repeats (55-57).

BRCA1 and *BRCA2* genes are altered in breast and ovarian cancers (58) by several mechanisms, including mutation, copy number and epigenetic silencing as a result of DNA hypermethylation. Sequence alterations in *BRCA1* and *BRCA2* are evident in over 20% of inherited breast cancer cases, and account for 60-80% lifetime risk of developing breast cancer and 20-40% risk of developing ovarian cancer (59). *BRCA1* and *BRCA2* mutations account for 50-70% of sporadic breast cancer cases. With these frequencies in mind, *BRCA1* and *BRCA2* inactivation correlates with genomic instability, chromosomal translocations and pronounced insertions and deletions. BRCA1 is involved in double strand break repair and is involved in guidance of the cell at the G2/S checkpoint. In addition, BRCA1 is involved in several complexes that activate or repress cell cycle arrest, DNA repair and anti-apoptotic processes (59). Inactivation of *BRCA1* as a result of mutation, deletion and silencing due to promoter DNA hypermethylation can occur on individual alleles in ovarian and breast cancers (60,61). Indeed, a population-based study of *BRCA1* DNA hypermethylation in breast tumors showed a positive correlation of *BRCA1* DNA methylation with lower survival rates (62).

Accompanying the concept of a select set of genomic alterations that drive tumorigeneisis is the concept of oncogenic addiction for cancer cell survival. As originally described by Weinstein, oncogenic addiction is thought of as the dependence of the cancer cell on a single oncogenic pathway for survival and the maintenance of the highly proliferative state (63). Interestingly, oncogenic addition supports the notion that targeting these specific addicted pathways can lead to efficacious therapeutic treatments, as these pathways are not active in normal cells. Examples of oncogenes that result in oncogenic addiction are *ABL*, *BRAF*, *EGFR*, *HER2*, *KIT*, *MET*, *MYC*, *RAS*, and others, across several forms of human cancer (64). Similarly, an addiction to the absence of tumor suppressor genes also exists in human cancer cells (65). Since tumor suppressor genes are inactivated in many human cancer types, re-activating tumor suppressor genes can also lead to deleterious results for cancer cell survival. Examples of tumor suppressor genes that foster oncogenic addiction when silenced include *DLC1*, *FHIT*, *PTEN*, *TP53* and *WWOX* (65).

The theme of cancer gene addiction can also be applied to DNA methylation, in that DNA methylation of a select set of genes in the cancer genome is absolutely essential for cancer cell growth and survival. In a report De Carvalho and colleagues (66), performed genome-scale DNA methylation analyses of human cancer cells deficient for one or more DNMTs in order to identify those genes that require DNA methylation for cancer cell survival. Indeed, the DNA methylation status of interleukin-1 receptor-associated kinase 3 (*IRAK3*) is cancer-specific, and correlated with reduced gene expression in cancer cells. Interestingly, IRAK3 inhibits MAPK, NFkB and STAT3 signaling pathways, all of which are activated in several cancer types. *IRAK3* promoter undergoes cancer-specific DNA hypermethylation and reduced gene expression in primary tissues across multiple cancer types. Therefore, *IRAK3* is an example of an epigenetic driver gene whose DNA methylation is essential for cancer cell survival.

Technological advancements of DNA methylation characterization

Restriction enzyme and PCR-based assays

The discovery of novel targets of cancer-specific DNA methylation and subsequent genome-wide characterization of human cancer methylomes is directly related to technological advancements in measuring DNA methylation changes [reviewed in (12,67)]. Initially, DNA methylation levels were measured globally using high-performance liquid chromatography (HPLC), which can separate cytosine from 5-mC nucleosides. For this reason, 5-mC has been labeled as the 5th base. However, HPLC only can quantitate global levels, but not at specific regions of the genome. The use of methylation-sensitive and methylation-insensitive DNA restriction enzyme isoschizomers (*Hpa*II versus *Msp*I) with PCR in assays such as combined bisulfite restriction analysis (COBRA), *Hpa*II tiny fragment enriched by ligation-mediated PCR (HELP) and methylated CpG island amplification (MCA) provided the ability to study DNA methylation changes at individual CpG sites, however, the surveyed CpG dinucleotides were limited to those that located are at the restriction sites of such enzymes (68-70).

Exploratory screening methods of biomarker discovery include methylation-sensitive arbitrarily primed PCR (MS-AP-PCR), amplification of intermethylated sites (AIMS) and restriction landmark genomic sequencing (RLGS), all of which utilize methylation-sensitive and methylation-insensitive restriction enzyme isoschizomer digestion (71-73). In MS-AP-PCR, genomic DNA digestion is followed by PCR with random CpG-rich primers and gel electrophoresis to identify aberrantly methylated regions that are then characterized by DNA sequencing. The AIMS assay is similar with respect to restriction enzyme treatment, but the methylated DNA ends are then ligated to adapters, and finally amplified by PCR using primers towards the adapter sequences. Like AP-PCR, AIMS PCR products are fractionated by gel electrophoresis to identify differentially methylated fragments that are subsequently sequenced. In RLGS, the digested DNA regions are fractionated on two-dimensional gels to isolate methylated and unmethylated loci. While these assays identify DNA hyper- and hypomethylated loci as biomarkers of disease, they may not be specifically targeted to promoter regions, and therefore may not be efficient in identifying regions that correlate with expression changes.

Since 5-mC marks are lost with PCR amplification of genomic DNA, DNA methylation assays were slower to develop as compared to assays to identify DNA sequence alterations. However, chemically treating genomic DNA with bisulfite results in the conversion of unmethylated cytosines to uracil (and thymine during PCR), while methylated cytosines are unaffected. Therefore, DNA methylation can be interpreted via cytosine versus thymine sequence differences (12). Bisulfite-mediated techniques were subsequently developed, such as candidate gene bisulfite sequencing, methylation-specific PCR (MSP), quantitative MSP (qMSP), MethyLight, pyrosequencing (PSQ) and methylation-sensitive single nucleotide primer extension (MS-SNuPE), in order to rapidly interrogate the DNA methylation status of candidate gene regions in large numbers of primary cancer tissues and cell lines (74-78).

MSP and MethyLight assays provide quick and efficient means for individual research laboratories to measure DNA methylation of virtually any candidate gene region. Gel-based MSP is largely a qualitative measure of DNA methylation, while qMSP and MethyLight are quantitative assays due to the inclusion of SYBR green and non-extending TaqMan fluorescent probes, respectively. In both instances, MSP and MethyLight technologies interrogate regions of concordant DNA methylation across 100-500 base pair PCR amplicons (74,77).

Microarray and next-generation sequencing applications

One caveat of candidate-gene technologies is the requirement of knowing the specific gene regions to interrogate. The union of the release of the human genome reference sequence with the development of microarray and next-generation sequencing technologies has provided the ability for rapid identification of aberrantly methylated candidate gene regions in human cancers. In addition, the combination of methylation-sensitive restriction enzymes, PCR and array hybridization in assays such as methylated CpG island amplification and microarray (MCAM) and differential methylation hybridization (DMH) provide genome-scale analyses of aberrantly methylated DNA regions in cancer tissues and cell lines (79,80).

Another approach for performing genome-scale methylome profiling utilizes enrichment in 5-mC content using antibodies directed towards methylated DNA (MeDIP) and methylated MBDs followed by hybridization to high-density DNA sequence arrays in order to identify hypermethylated genomic DNA regions. These include MeDIP-chip, MBD-chip, the methylated-CpG island recovery assay (MIRA) and comprehensive high-throughput

arrays for relative methylation (CHARM) (81-84). This approach has identified cancer-specific DNA methylation events, however, it is not applicable to large numbers of samples and may not accurately identify methylated DNA regions as compared to bisulfite-based methods.

Three Illumina DNA methylation BeadArray platforms, GoldenGate, Infinium HumanMethylation27 (HM27) and Infinium HumanMethylation450 (HM450), provide genome-scale DNA methylation detection of 1,536, 27,578 and 482,421 CpG dinucleotides, respectively, with the ability of surveying DNA methylation levels of large numbers of samples (85-89). Currently, the Illumina HM450 BeadArray is the only commercially available DNA methylation array platform, and is regarded as a cost-effective, high-throughput method for biomarker and tumor subtype identification. The HM450 platform can also be used in conjunction with nucleosome positioning (accessible) and oxidative bisulfite based 5-hmC profiling assays to obtain integrated views of the cancer epigenome (90-93).

Next-generation based sequencing approaches (-seq), including MBD-seq, MeDIP-seq, whole-genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS), have become more cost-effective and therefore increasingly utilized to obtain more comprehensive DNA methylation maps. Using WGBS, DNA methylation information can be obtained for nearly all of the 28 million total CpG sites in the human genome (26,32,33). However, WGBS requires substantial sequencing depth (4-30× genome coverage) to obtain high-quality and interpretative data, and is challenging with respect to mapping CpG-rich sequences and repetitive element regions of the genome. Alternatively, RRBS involves the use of methylation-specific restriction enzyme isoschizomers (*Hpa*II versus *Msp*I), followed by library construction, for profiling of approximately 1% of the human genome, thereby reducing the overall required sequencing while determining the DNA methylation status of 1-2 million CpG dinucleotides, mostly in CpG islands and gene promoters (94,95). Finally, the recently reported NOMe-seq (nucleosome occupancy and methylation) technology displays the ability to concurrently determine both DNA methylation and nucleosome occupancy (96).

Sensitive detection of human cancers using DNA methylation-based approaches

Since cancer-specific DNA methylation alterations are stable and present in all forms of human cancer, DNA methylation biomarkers have a promising utility for cancer diagnostics, disease monitoring, treatment response, as well as prediction of disease risk and survival. With these applications in mind, there is tremendous interest in identifying cancer-specific events for early detection purposes, as cancer-derived DNA is present in the bloodstream of cancer patients. Additional bodily fluids, including urine sediment, sputum and fecal matter, also represent promising media for capturing and quantifying cancer-specific genomic alterations.

Colorectal cancer detection using the *SEPT9* DNA methylation marker in cell-free DNA isolated from plasma has shown great promise as an early detection biomarker. *SEPT9* DNA methylation was identified after a screening cancer-specific DNA hypermethylation using MS-AP-PCR and MCA approaches (97). The *SEPT9* assay displays a mean 75% sensitivity and 87% specificity after testing of thousands of samples using a variety of protocols in the United States and Europe [summarized in (98)]. Specifically, the second iteration of the assay displayed mean detection sensitivities of 67%, 83%, 84% and 100% for stage I, II, III and IV colorectal cancers, respectively (99). Moreover, the assay is positive in nearly 30% of advanced adenomas, with 58% sensitivity and 82% specificity (99). Overall, the *SEPT9* DNA methylation assay is currently distributed as commercially available diagnostic kits under the names Epi proColon (Epigenomics), mS9 (Abbott) and ColoVantage (Quest) (100,101).

Cancer-derived, methylated DNA detection in urine sediment is also a promising detection method for bladder and prostate cancers. Due to the large volume of urine available and the ease of sample collection and DNA isolation, urine is an attractive media for disease detection, surveillance, recurrence and response to treatment. Indeed, Su *et al*. recently described a panel of three markers comprised of *SOX1* and *IRAK3* DNA hypermethylation and *LINE-1* repetitive element DNA hypomethylation that predict tumor recurrence superior to cytology and cystoscopy, which are regarded as the gold standard for bladder cancer surveillance and monitoring (102). Several DNA methylation-based biomarkers of prostate cancer have been identified in urine, including *APC*, *GSTP1*, *HOXD3* and *TGBR2* (103,104), while detection of *RASSF1* DNA hypermethylation in urine correlated with prostate tumor recurrence (105).

Digital PCR methods have also improved the discovery of cancer-associated DNA methylation alterations. Bisulfite-

based PCR assays performed in individual assay wells are largely limited in detection sensitivity and resolution. In contrast, digital PCR-based assays, in which DNA molecules are PCR-amplified over multiple reaction wells, allow for an accounting of individual methylated DNA molecules (106). This is accomplished since template and non-template molecules are sequestered into individual reaction wells, thereby reducing PCR inhibition and improving the signal to noise ratio.

One application of digital PCR to DNA methylation technology is Digital MethyLight, which results in improved detection sensitivity and quantitative accuracy of individual methylated template DNA molecules (107). Digital MethyLight is not only compatible with conventional real-time PCR platforms, but also with microfluidic and digital droplet PCR platforms (107,108). Digital MethyLight was first utilized to quantitate individual cancer-derived DNA hypermethylation events in *CLDN5*, *FOXE1* and *RUNX3* in serum of breast cancer patients (107). Moreover, Campan and colleagues identified DNA methylation of *IFFO1* in ovarian cancer patients after Illumina Infinium DNA methylation screening of ovarian tumors and non-tumor tissues (109). Interestingly, tumor-derived *IFFO1* detection in patient serum correlated with CA-125 levels currently used for determining disease burden and relapse after tumor resection (109). Similarly, Illumina Infinium DNA methylation screening was used to identify *THBD* and *C9orf50* DNA methylation in colorectal tumors, as well as their potential utility as early-detection markers of colorectal cancer in plasma and serum (110). *THBD* and *C9orf50* DNA methylation outperformed carcinoembryonic antigen (CEA), a clinically-approved marker for colorectal cancer detection in blood (111,112), in terms of tumor detection sensitivities (110).

DNA methylation characterization of human cancers from multi-institutional consortia

As a result of technological advancements in surveying human cancer genomes, molecular profiling of large numbers of primary tumors in order to paint integrative molecular portraits of individual cancer types, is currently attainable. These integrated views, coupled with patient clinical information and large sample numbers, can be utilized for identifying novel molecular-based tumor subgroups and therapeutic approaches. However, determining these features in a genome-wide manner is challenging for several reasons, including the costs of generating and analyzing molecular data, the requirement for stratifying molecular data for large numbers of samples as functions of age, gender, race/ethnicity, environmental exposures and family history. Moreover, genomic features of human cancers also require tissue-specific interrogations, thereby increasing experimental complexity and scope. Substantial laboratory and bioinformatics expertise is also essential, and therefore, is challenging to complete in isolation. Genomic, epigenomic and transcriptomic alterations do not exist in isolation, since the cancer genome typically displays an abundance of structural and regulatory aberrations, and therefore, integrative methods and approaches are required to obtain a complete picture of the cancer genome. The cancer genome is not only confounded by the challenges of identifying linkages between DNA sequence, epigenetics and expression data, but also the issues of genetic/chromosomal instability, tumor cell heterogeneity, multilevel selection and the complex nature of cancer evolution (113-115).

In recognizing these challenges, several multi-institutional consortia have been organized for efficient molecular characterization and analyses. These include Encyclopedia of DNA Elements (ENCODE) (116-118), International Cancer Genomics Consortium, International Human Epigenome Consortium (119), NIH Roadmap Epigenomics Initiative (120-122) and The Cancer Genome Atlas (TCGA) have been vital in identifying genomic and epigenomic alterations in normal tissues, cancer tissues and cancer cell lines.

In particular, TCGA has been prolific in generating and analyzing genomic, epigenomic and transcriptomic data for over 11,000 primary tumors and 1,000 normal-adjacent tissues spanning 30 human cancer types (*Table 1*) (137). In order to detect low-penetrating genomic alterations, TCGA accrued 500 tumors for most tumor types. Molecular profiling includes mutation detection (whole-genome and whole-exome sequencing), gene expression profiling (RNA-seq and miRNA-seq), somatic copy number alterations (single nucleotide polymorphism arrays), and DNA methylation profiling (Illumina BeadArrays) (85-88). The unique and powerful aspects of the TCGA collections stem from large sample numbers, centralized pathological review, selection of samples with a high fraction of tumor nuclei, genomic characterizations over multiple assay platforms using nucleic acids isolated from the same tissue samples and data integration for high-level pathway interpretations. TCGA has published high profile, integrative analyses of 16 individual tumor types (45,50,60,61,123-135), and is

Table 1 TCGA tumor types selected for genomic characterization

Tumor type	TCGA tumor abbreviation	TCGA consortium publication reference	Analyzed in pan-cancer-12 analyses[†]
Adrenocortical carcinoma	ACC		
Bladder urothelial carcinoma	BLCA	(123)	Yes
Breast invasive carcinoma	BRCA	(61)	Yes
Cervical squamous cell carcinoma and endocervical adenocarcinoma	CESC		
Colon and rectal adenocarcinoma	COAD & READ	(50)	Yes
Lymphoid neoplasm diffuse large b-cell lymphoma	DLBC		
Esophageal carcinoma	ESCA		
Glioblastoma multiforme	GBM	(45,124-126)	Yes
Head and neck squamous cell carcinoma	HNSC	(127)	Yes
Kidney chromophobe renal cell carcinoma	KICH	(128)	
Kidney renal clear cell carcinoma	KIRC	(129)	Yes
Kidney renal papillary cell carcinoma	KIRP		
Acute myeloid leukemia	LAML	(130)	Yes
Lower grade glioma (brain)	LGG		
Liver hepatocellular carcinoma	LIHC		
Lung adenocarcinoma	LUAD	(131)	Yes
Lung squamous cell carcinoma	LUSC	(132)	Yes
Mesothelioma	MESO		
Ovarian serous cystadenocarcinoma	OV	(60)	Yes
Pancreatic adenocarcinoma	PAAD		
Pheochromocytoma & paraganglioma	PCPG		
Prostate adenocarcinoma	PRAD		
Sarcoma	SARC		
Skin cutaneous melanoma	SKMC		
Stomach (gastric) adenocarcinoma	STAD	(133)	
Thyroid carcinoma	THCA	(134)	
Uterine corpus endometrioid carcinoma	UCEC	(135)	Yes
Uterine carcinosarcoma	UCS		
Uveal melanoma	UVM		

[†], as described in reference (136). TCGA, The Cancer Genome Atlas.

currently pursuing similar analyses for the remaining tumor types, as well integrated analyses of molecular data across multiple cancer types (136,138).

CpG island methylator phenotypes (CIMPs) as unique and clinically relevant tumor subgroups

Currently, there is wide interest in identifying and characterizing novel DNA methylation-based subgroups of tumors with the goal of determining their relationships to clinical features and other genomic alterations for diagnostic and treatment purposes. In 1999, Toyota and colleagues first described a distinct subset of human colorectal cancers with extensive DNA hypermethylation of a subset of CpG islands that remained unmethylated in the remaining colorectal tumors (139), and are therefore unique from general cancer-specific DNA methylation for a specific tumor type. These tumors, representing

approximately 15% of all colorectal cancers, are classified as positive for a CIMP. Colorectal CIMP-positive tumors, currently referred to as CIMP-high (CIMP-H), are enriched for *BRAF*^{V600E} mutations, microsatellite instability, *MLH1* DNA hypermethylation, a hypermutated profile and the absence of copy number alterations and *KRAS* mutations. Colorectal CIMPs are largely located in the proximal colon, are more prevalent in patients of older age and female gender, family history and demonstrate improved survival (44,50,140-143). In addition, CIMP status in female patients positively correlated with increased pack years of smoking and obesity (144).

In contrast to CIMP-H tumors, an additional CIMP-like subgroup, described as CIMP-low (CIMP-L) and CIMP2, has been identified in a subset of colorectal tumors (50,145,146). CIMP-L tumors display attenuated CIMP-associated DNA methylation, mixed microsatellite stability, extensive copy number alterations and enrichment in *KRAS* mutations (44,50,143,145). TCGA confirmed the CIMP-H and CIMP-L subgroups, along with their associations to the previously described molecular features (50).

The TCGA consortium was instrumental in characterizing a novel CIMP subgroup (G-CIMP) in glioblastoma (grade IV glioma), which is present in approximately 15% of GBM cases, and is nearly completely correlated with a specific heterogeneous point mutation in the *IDH1* gene (*IDH1*^{R132H}) (45). All TCGA primary GBM tumors with an *IDH1* mutation (*IDH1*^{MUT}) are G-CIMP, however, a small number of G-CIMP tumors are wild type for *IDH1* (*IDH1*^{WT}). G-CIMP tumors are enriched for *TP53* alterations, reduced copy number alterations, and correlated with improved survival and younger patient age.

The association of G-CIMP with *IDH1* mutations connected two seemingly disparate aspects of cellular biology. While IDH1^{WT} functions in the citric acid cycle by converting isocitrate to alpha-ketoglutarate (α-KG) [reviewed in (147)], IDH1^{MUT} also catalyzes the conversion of α-KG to D-2-hydroxyglutarate (2-HG), and importantly, inhibits TET function and subsequent DNA demethylation. TET inhibition as a result of IDH1^{MUT} supports the hypermethylated DNA landscape in G-CIMP tumors, and this landmark mechanistic discovery has great clinical promise, not only for diagnostic purposes, but also the potential use of epigenetic therapies for treating patients with G-CIMP tumors.

TCGA also recently characterized two gastric cancer CIMP subgroups report (133). The first, *gastric CIMP*, displays a colorectal-like CIMP DNA methylation profile, together with hypermutation, MSI and *MLH1* epigenetic silencing. The second set of gastric CIMP tumors, *EBV-CIMP*, is associated with EBV infection, is present in nearly 10% of gastric cancer cases, and display extensive CIMP-DNA methylation even beyond that of the Gastric CIMP group. EBV-CIMP is also enriched for mutations of the chromatin remodeler *ARID1A*, as well as *CDKN2A* silencing. While the correlation of EBV infection with the EBV-CIMP subgroup is currently unknown, this represents another powerful connection between a novel DNA methylation-based subgroup and unique genomic, clinical and biological features.

CIMP subgroups of breast (B-CIMP) (61) and endometrial (E-CIMP) (135) cancers have also been characterized. The TCGA integrated breast cancer report showed that B-CIMP tumors were positive for estrogen and progesterone receptor expression, as well as for HER2 expression, and were enriched for epigenetic silencing of genes in the Wnt-signaling pathway (61), as also described for colorectal tumors (50). E-CIMP tumors are similar to colorectal CIMP tumors in that both display hypermutation, *MLH1* promoter DNA hypermethylation, MSI, and the absence of both *TP53* somatic mutations and copy number alterations (50,135). Finally, it should be noted that neither E-CIMP nor B-CIMP tumors harbor *BRAF*^{V600E} or *IDH1* mutations, as described in colorectal and glioma CIMP tumors, respectively, pointing to the hypothesis that individual CIMPs may arise from several possible overlapping and non-overlapping molecular mechanisms.

Cancer-associated DNA methylation alterations as targets for epigenetic-based therapeutics

An important aspect of cancer-associated epigenetic alterations that should not be overlooked is that unlike somatic mutations and copy number alterations, DNA methylation and histone modifications are reversible, and therefore, aberrant epigenetic profiles can be corrected using inhibitors to DNMTs and histone modifiers. The first-generation epigenetic inhibitors based on DNMT inhibition, 5-azacytidine (5-Aza-CR, Vidaza) and 5-aza-2'-deoxycytidine (5-Aza-CdR, decitabine, dacogen), have been approved for treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) patients (148,149). In addition, a second-generation inhibitor, zebularine (pyrimidin-2-one β-D-ribofuranoside), also inhibited DNA

methylation after oral administration in both *in vitro* and *in vivo* settings (150,151).

The treatment of cell line models of human breast, colon and lung tumors with low doses of vidaza resulted in the re-expression of genes specific for immune response and cancer testis antigens, including interferon signaling, antigen processing and cytokines/chemokines (36,152,153), and down regulation of oncogenic signaling pathways, such as MYC and metabolic pathways (40). Moreover, combinatorial treatments of histone deacetylase (HDAC) inhibitors, together with low doses of vidaza over several treatment cycles, also induced gene expression of genes involved in cell cycle regulation, cytoskeletal organization and DNA damage response (154). Indeed, clinical trials in which vidaza and the HDAC inhibitor entinostat were administered to patients with recurrent non-small cell lung cancer (NSCLC) were overall well tolerated, and showed prolonged patient survival (155).

In support of advancing clinical trials with novel epigenetic-based therapies, the recently-introduced DNA methylation inhibitor S110 (AzapG), a dinucleotide consisting of 5-aza-2'-deoxycytidine connected upstream to a deoxyguanosine, was shown to be an effective DNA methylation inhibitor by resisting cytidine deaminase degradation in both *in vitro* and *in vivo* settings, and showed improved stability and reduced toxicity compared to aza-substituted mononucleosides (156,157), Currently, S110 is being analyzed for potential clinical utility in phase II clinical trials for treatment of ovarian, liver and colorectal cancers, as well as AML, MDS, either alone, or in combination with other therapeutic agents.

Conclusions

DNA methylation is a complex regulatory element, and it is not surprising that DNA methylation is dysregulated in virtually every tumor type. As a result, gene expression and subsequent signaling pathways are affected, and implicates DNA methylation alterations as early events in tumorigenesis. Importantly, DNA methylation is now looked upon as a major genomic feature of human cancers, together with somatic mutations and copy number changes. Indeed, genes are inactivated by individual types of alterations, as well as by multiple alterations, satisfying Knudsen's two-hit hypothesis. In addition, DNA methylation aberrancies represent promising cancer therapeutic targets, since DNA methylation profiles are reversible through the use of inhibitors. This feature of cancer epigenomics is important, as DNA methylation inhibitors can be utilized for exploiting addictions to oncogenes and the absence of tumor suppressors. DNA methylation markings can also have clinical utility in their development as cancer biomarkers. DNA methylation-based biomarkers have been described in several reports as indicators of tumor presence, as well as predictors of recurrence, progression. Moreover, DNA methylation biomarkers can be detected in several biological fluids, such as blood, sputum and urine, for early detection of human cancers.

Finally, DNA methylation alterations can now be characterized across the genome in large numbers of primary tissues using next-generation sequencing and microarray methods. Moreover, multi-institutional consortia, including ENCODE, the Epigenetics Roadmap Initiative and TCGA, have been instrumental in generating diverse sets of epigenomic data on large numbers of normal and tumor cell lines and tissues. These publically available datasets have been important in cancer epigenomic profiling and validation efforts, as well as identifying tumor subgroups. In support of this, DNA methylation based subgroups of individual tumor types have been characterized. These are primarily classified as CIMP, and represent subsets of tumors with accentuated cancer-specific DNA methylation profiles. Importantly, CIMPs are clinically relevant, and correlate with patient age, survival and other genomic features.

In summary, DNA methylation represents an important aspect of cancer genomics, based on their roles in gene regulation, biomarkers and therapeutic targets. The improvement of whole-genome sequencing technologies, especially in determining the epigenomic profiles of large, population-based tumor collections, as well as single cells and cell-free tumor-derived DNA in biological fluids, will provide a wealth of information for obtaining higher-resolution maps of the cancer epigenome for refined cancer detection, monitoring, surveillance and targets of therapeutic efficacy.

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